Surfactants and the attachment of *Pseudomonas aeruginosa* to 3CR12 stainless steel and glass

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Abstract

Five non-ionic and three anionic surfactants were evaluated using 4'6-diamidino-2-phenylidole (DAPI) staining, scanning electron microscopy (SEM) and spectrophotometry for their efficacy in preventing adhesion and removing *Ps. aeruginosa* attached to 3CR12 stainless steel coupons and glass. All the surfactants tested gave more than 90% inhibition of adhesion to the surfaces tested with no significant difference between the effectivity of the different anionic surfactants ($p > 0.18$) nor between the effectivity of the non-ionic surfactants ($p > 0.16$). The non-ionic and anionic surfactants resulted in more than 80% and 63% removal of attached *Ps. aeruginosa* cells, respectively. The non-ionic surfactants were significantly more effective in removing attached bacteria, than the anionic surfactants ($p < 0.001$). The prevention of attachment of *Ps. aeruginosa* cells to a glass surface, using the surfactants, was also monitored spectrophotometrically. There was no significant difference ($p = 0.437$) when comparing the DAPI - staining technique with spectrophotometric evaluations.

Introduction

Adhesion to surfaces is a common and well-known characteristic of micro-organisms in oligotrophic habitats (Zobell, 1943). This adhesion and subsequent metabolism lead to the formation of biofilms (McCoy et al., 1981). Bacterial biofilms promote increased biomass deposition (Whitekettle, 1991), resulting in fluid flow resistance, loss of heat exchange and microbially induced corrosion in industrial water cooling systems (Marshall, 1992).

Industries control unwanted biofilms, with varying degrees of success, by using biocides (Marshall, 1992; Cloete et al., 1998). The use of biocides, especially chlorine, in water reticulation and heat-exchange systems is effective only if the biofilm is removed manually. Chlorination of a mature biofilm is usually unsuccessful because the biocide only reacts with the outer portion of the biofilm, leaving a healthy and substantial bacterial community on the surface that rapidly regrows (Marshall, 1992). Bacteria within biofilms develop increasing resistance to non-oxidising biocides on repeated dosing (Cloete et al., 1992). Brözel and Cloete (1992) found that non-oxidising biocides also induced cross-resistance to other non-oxidising biocides.

More recently, surface-active compounds (surfactants) have been employed to prevent bacterial adhesion to surfaces. Currently there is no evidence that surfactants will have any mutagenic effects on bacteria, or that micro-organisms could become resistant to the action of surfactants, as in the case of biocides (Russel, 1990; Brözel and Cloete, 1992). Unfortunately, little published information is available on the effectivity of different biodispersants (surfactants) against bacterial attachment (Lutey, 1995). According to Paul and Jeffrey (1985), dilute surfactants completely inhibited the attachment of estuarine and marine bacteria. Surfactants result in uniform wetting of the surface to be treated and have an additional cleaning effect (Cloete et al., 1992; Lutey, 1995). Whitekettle (1991) found a correlation between the ability of a surface-active compound to lower surface tension and its ability to prevent microbial adhesion. White and Russel (1992) classified surfactants according to the ionic nature of the hydrophilic group viz. anionic, cationic, non-ionic and zwitterionic.

The aim of this study was to use DAPI staining, scanning electron microscopy (SEM) and spectrophotometry to monitor the adhesion of *Ps. aeruginosa* to stainless steel and glass surfaces and to use these methods to monitor the removal of a mature biofilm from a stainless steel surface using non-ionic and anionic surfactants (biodispersants).

Materials and methods

Organism used

Pseudomonas aeruginosa isolated from a cooling water system was used for all the experiments (Brözel and Cloete, 1992).

Surfactants used

Non-ionic and anionic surfactants were obtained from South African suppliers (Table 1). The dosing concentration of the surfactants was 20 $mg \cdot l^{-1}$ according to manufacturer instructions.

Experimental procedures

A continuous flow - through system (Jacobs et al., 1996) and a modified Pedersen device (McCoy et al., 1981) were used to determine the prevention of adhesion and biofilm removal of *Ps. aeruginosa* on a stainless steel surface and on glass.

A wild strain of *Pseudomonas aeruginosa* isolated from a cooling water system and identified in a previous study was used (Brözel and Cloete, 1992). A modified Pedersen device (McCoy et al., 1981) and a flow-through tube were connected in series with a peristaltic pump, which in turn was connected to a 4 l reservoir. *Pseudomonas aeruginosa* was cultured in 200 ml R2A broth (Reasoner and Geldreich, 1985) for 24 h at room temperature. Of this culture 20 ml were used to inoculate the reservoir containing 4 l R2A medium. The flow rate through the system was 1.8 $ml \cdot h^{-1}$.

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TABLE 1
***Pseudomonas aeruginosa* adhesion to the 3CR12 stainless steel coupons in the presence of anionic and non-ionic surfactants at a concentration of 20 mg·l^{-1}**

Treatment	Supplier	Number of attached cells/cm^{-2}	Standard deviation	Inhibition of attachment (% of control)
Control	1.24 x 10^5	3.33 x 10^2		
Anionic surfactants:				
Product C1	Chemserve	7.88 x 10^3	3.70 x 10^2	93.64%
Product C35	Chemserve	7.86 x 10^3	2.90 x 10^2	93.66%
Product B3	Chemserve	7.89 x 10^3	2.60 x 10^2	93.63%
Non-ionic surfactants:				
Product B6	Buckman	6.54 x 10^3	4.00 x 10^2	94.73%
Product C33	Chemserve	6.56 x 10^3	6.10 x 10^2	94.71%
Product C17	Chemserve	6.56 x 10^3	6.60 x 10^2	94.71%
Product C24	Chemserve	6.58 x 10^3	3.30 x 10^2	94.69%
Product A1	Anikem	6.53 x 10^3	4.50 x 10^2	94.73%

Sterile medium was continuously fed into the reservoir at a dilution rate of 240 ml·h^{-1}. The same amount of used medium and cells left the reservoir, keeping the cells in the same growth phase at all times and ensuring a constant concentration of 10^8 cells·ml^{-1} in the reservoir and the flow-through system.

The modified Pederson device was used because it allows the study of a relatively large surface area of undisturbed biofilm. The following additional modifications were made:

- the device was perspex, instead of steel;
- the size of microscope slides were used instead of microscope cover slips;
- only one test pile was used; and
- coupons were vertically installed instead of horizontally.

Twenty 3CR12 metal coupons were held in place in the device. Coupons, 75 x 27 x 1 mm in size were used for the DAPI-staining. Smaller coupons (25 x 27 x 1 mm) were used for the scanning electron microscopy studies. Before each experiment, the coupons were prepared as described by Wolfaardt et al. (1991). Prior to use, the device was cleaned with a soap solution, thoroughly rinsed, sterilised with 5% hydrogen peroxide for 10 min. and flushed with sterile distilled water to remove any residues.

Quantification of attached bacteria using 4,6-diamidino-z-phenylidole (DAPI)

DAPI staining was done as described in a previous study (Wolfaardt et al., 1991). The 75 x 27 x 1 mm coupons were removed from the Pedersen device and rinsed with sterile water as described for the SEM studies of biofilm formation and stained with DAPI for epifluorescence microscopy (Wolfaardt et al., 1991). Attached bacteria were counted under oil immersion using Epifluorescence microscopy. Ten randomly chosen microscope fields were counted under the 800 x magnification.

Spectrophotometric measurements: Absorbance of the bacteria attached to the glass tube was measured simultaneously with the removal of the coupons from the modified Pedersen device. The SQ 118 Spectrophotometer (Merck, PTY.LTD), adjusted to a wavelength of 550nm, was used. Before each measurement, a zero adjustment was done on the spectrophotometer using a clean tube filled with distilled water. Absorbance of the bacteria attached to the flow-through tube was determined after carefully rinsing and replacing the medium in the tube with sterile distilled water.

Scanning electron microscopy (SEM): Coupons (25 x 27 x 1mm) were removed, in duplicate, from the modified Pedersen device after 4, 8, 24, 32, 48 and 56 h, with a sterile forceps and replaced with a sterile coupon, in order to keep the flow constant. After removal the coupons were rinsed with sterile distilled water for 30 s to remove any unattached cells and then fixed for SEM by the following series of treatments: 2% gluteraldehyde (1 h); 0.175M phosphate-buffer (3 x 15 min); 50% ethanol (1 x 15 min); 70% ethanol (1 x 15 min); 90% ethanol (1 x 15 min) and 100% ethanol (3 x 15 min). The coupons were thereafter dried in a critical point dryer, mounted on studs and coated with gold plasma and examined using the Hitachi S-450 scanning electron microscope

The non-ionic and anionic surfactants were tested separately at the recommended concentrations (20 mg·l^{-1}). *Ps. aeruginosa* was cultured in 200 ml R2A broth (Reasoner and Geldreich, 1985) for 24 h at room temperature. Of this culture, 20 ml were used to inoculate the reservoir containing 4 l of R2A broth, and either prevention of adhesion or biofilm removal was studied.

In order to prevent adhesion of *Ps. aeruginosa* to the stainless steel coupons and the glass surfaces, the surfactants were introduced into the reservoir at the beginning of the experiment.

The experiment was allowed to proceed for 32 h, during which time the surfactants were fed continuously into the system. The flow rate in the system was 1.2 m·s^{-1}. Prevention of adhesion to the stainless steel surfaces was studied with DAPI and SEM, while spectrophotometry was used for the glass surface.

To study biofilm removal, the bacteria were allowed to adhere

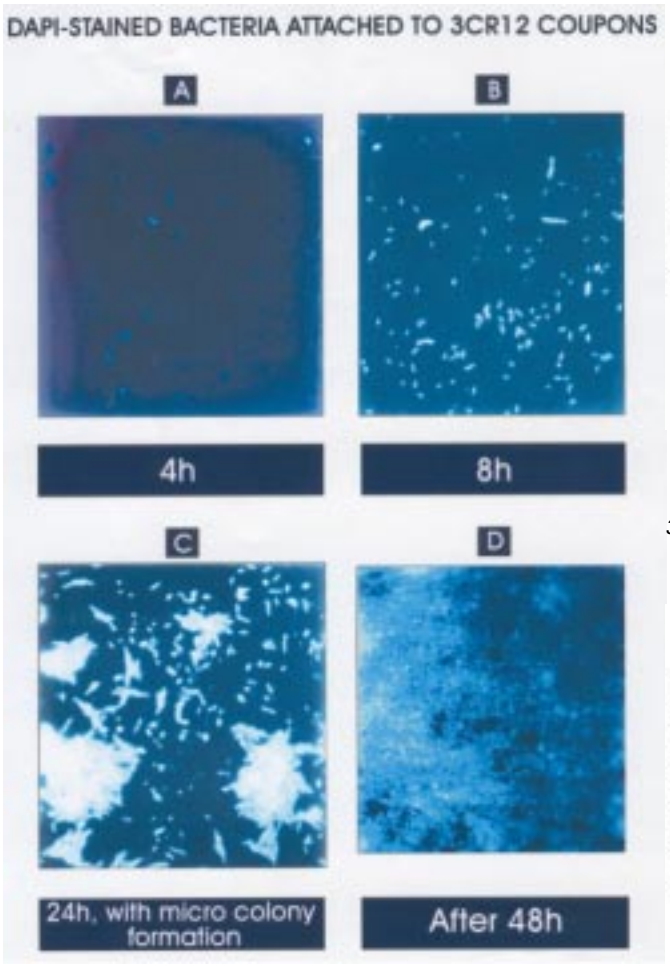

Figure 1
Biofilm formation by Ps. aeruginosa *over a 48 h period without any treatment (Epifluorescence microscopy 1000 x magnification)*

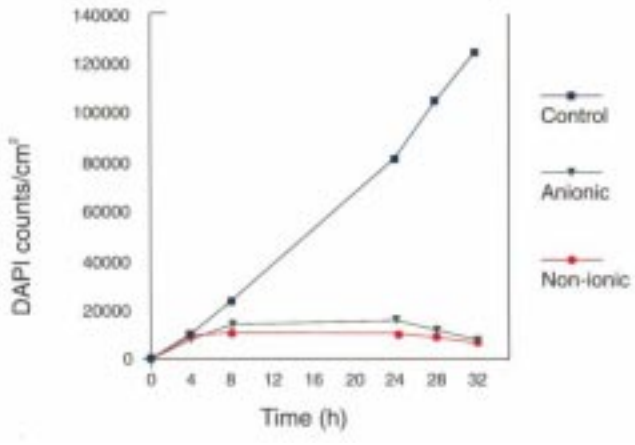

Figure 2
Number of DAPI-stained Ps. aeruginosa *cells adhering to the 3CR2 coupons over a period of 32h in the presence of 20 mg·l⁻¹ of surfactant*

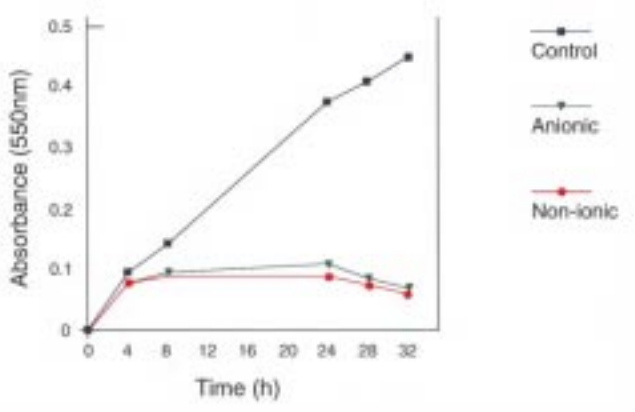

Figure 3
Absorbance measurements of Ps. aeruginosa *adherence to a glass tube over a period of 32 h in the presence of 20 mg·l⁻¹ of surfactant*

to the surface of the 3CR12 stainless steel coupons for 168 h, before commencing the dosing of the surfactants. The experiment was allowed to proceed for 32 h. Samples were removed before and after treatment and biofilm removal was determined with DAPI and SEM.

For both experiments, a control system, where no surfactants had been added, was included. All experiments were done in triplicate.

Expression and statistical analysis of results

The results are expressed as the mean value of the standard error means for each treatment. Levels of statistical significance were calculated using the students t-test.

Results

Adhesion of *Ps. aeruginosa* to 3CR12 coupons and a glass tube in the absence of surfactants

Ps. aeruginosa readily adhered to the 3CR12 coupons (Fig. 1) and the glass tube in the absence of the surfactants (control system) (Fig. 3). The number of bacteria adhering to the surfaces increased to 1.24×10^5 bacteria·cm^{-2} after 32h as determined using the DAPI-staining technique (Fig. 2). Absorbance measurements also indicated increased attachment of *Ps. aeruginosa* to the glass tube over time in the control system (Fig. 3).

Ps. aeruginosa adhesion to 3CR12 stainless steel coupons and a glass tube in the presence of surfactants

All the surfactants tested, inhibited *Ps. aeruginosa* adhesion to 3CR12 stainless steel significantly, when compared to the control (Figs. 2 and 3 and Table 1). The number of attached bacteria increased during the first 8 h of exposure in the presence of the non-ionic (product B6) and the anionic (product C1) surfactant (Fig. 2). During the next 16 h of exposure to the surfactants, the rate of bacterial adhesion slowed down and declined after 24 h (Fig. 2). After 32 h, the number of attached bacteria remained below 1×10^4 bacteria·cm^{-2} for all the surfactants tested, compared to the 1.24×10^5 bacteria·cm^{-2} of the control (Table 1 and Fig. 2). Anionic and non-ionic surfactant treatments resulted in an average of 93% and 94% inhibition of bacterial attachment respectively (Table 1, Figs. 2 and 3). There was no significant difference amongst the effectivity of the different anionic surfactants nor amongst the effectivity of

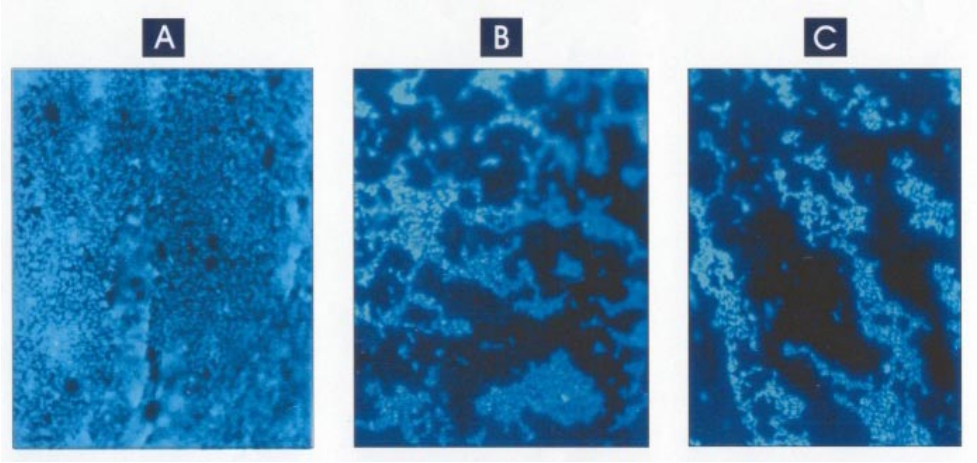

Figure 4
DAPI stains of a 168 h Pseudomonas aeruginosa *biofilm on 3CR12 coupons (A) after 168 h, (B) after treatment with the nonionic surfactant (Product B6) for 24h and (C) after treatment with the anionic surfactant (Product C1) for 24h. (Epifluorescence microscopy 1000 x magnification)*

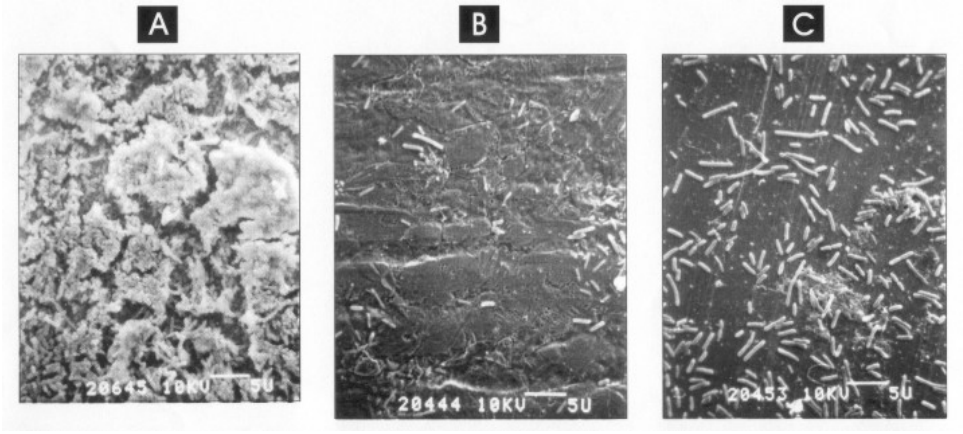

Figure 5
SEM photomicrographs of a 168 h Ps. aeruginosa *biofilm on a 3CR12 coupon (A) before treatment, (B) 24 h after continuous exposure to a non-ionic surfactant (Product B6) and (C) 24 h after continuous exposure to an anionic surfactant (Product C1). (Bars = 5µm)*

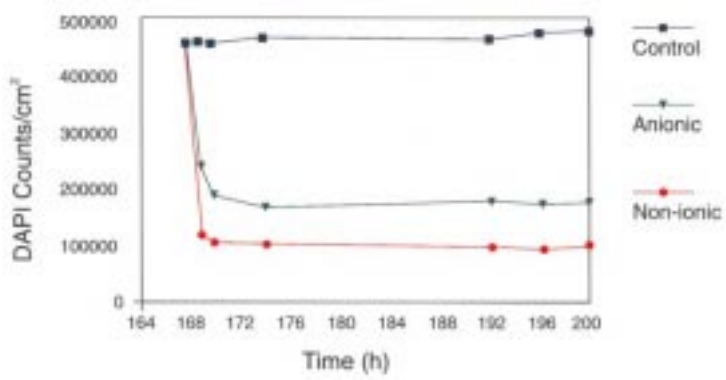

Figure 6
The number of DAPI-stained Ps. aeruginosa *cells adhering to the 3CR12 coupons over a time period of 32 h after treatment with an anionic surfactant (C1) and a nonionic surfactant (B6) at 20 mg·l⁻¹. Treatment started after cells were allowed to attach for 168 h.*

the non-ionic surfactants.

The absorbance measured for bacterial biofilm formation after exposure of the system to the anionic (C1) and non-ionic (B6) surfactant for 32 h was 0.069 and 0.060 respectively (Fig. 3). The absorbance for the control system after 32 h was 0.445 (Fig. 3). The inhibition of bacterial attachment to the glass tube after treating the system for 32 h was therefore 86.5% and 84.4% respectively for the products B6 and C1.

The percentage inhibition of bacteria adhering to the coupons after exposing the system to product B6 and C1 over a time period of 32 h using the DAPI-staining technique and spectrophotometric evaluations, was compared. There was no significant difference ($p = 0.437$) between the results obtained using these different methods.

Removal of attached bacteria

The non-ionic surfactants were significantly more effective in removing pre-attached cells from the stainless steel coupons than the anionic surfactants (Figs. 4, 5 and 6). These results were

TABLE 2
Removal of pre-attached *Pseudomonas aeruginosa* to 3CR12 coupons, by exposing the biofilm to anionic and non-ionic surfactants at a concentration of 20 mg·l^{-1} for 32h. The DAPI-staining technique was used for monitoring adhesion

Treatment	Number of attached cells/cm^2	Standard deviation	% Removal
Control	4.68 x 10^5	3.37 x 10^2	
Anionic surfactants:			
Product C1	1.68 x 10^5	3.33 x 10^2	64.10%
Product C35	1.71 x 10^5	5.77 x 10^2	63.46%
Product B3	1.70 x 10^5	4.68 x 10^2	63.67%
Non-ionic surfactants:			
Product B6	9.15 x 10^4	2.07 x 10^2	80.45%
Product C33	9.14 x 10^4	1.15 x 10^2	80.47%
Product C17	9.16 x 10^4	3.07 x 10^2	80.43%
Product C24	9.13 x 10^4	1.06 x 10^2	80.49%
Product A1	9.18 x 10^4	2.48 x 10^2	80.38%

obtained after subjecting a mature (168h) *Ps. aeruginosa* biofilm to the surfactants. All the surfactants tested resulted in a significant decrease in bacteria attached to the 3CR12 stainless steel coupons (Table 2).

The anionic surfactant (product C35) produced a 63.46% decrease in attached cells compared to the control. There was an 80.45% decrease in the number of attached bacteria when the non-ionic surfactant (product B6) was used (Figs. 4, 5 and 6). After the initial decrease in attached cells, the number of bacteria adhering to the surfaces remained constant, until dosing was stopped, upon which the number of attached bacteria increased.

The non-ionic and anionic surfactants effected a decrease of on average 80.44% and 63.74% in attached bacteria, respectively. There was no significant difference between the effectivity of the various anionic surfactants tested nor the effectivity of the non-ionic surfactants.

Discussion

All the non-ionic and anionic surfactants had anti-adhesive effects on bacterial attachment. There was no significant difference in the effectivity of the different anionic surfactants nor amongst the effectivity of the different non-ionic surfactants. The non-ionic surfactants were more efficient in preventing *Ps. aeruginosa* adherence to the surface, than the anionic surfactants. This difference in effectivity is of no practical value for industrial water systems. These results compared well with the results of Whitekettle (1991) who found that the surfactants produced more than 90% inhibition of microbial adhesion. According to Whitekettle (1991) non-ionic surfactants had the greatest efficacy as inhibitors of microbial adhesion. Compounds capable of reducing surface tension by 20 mN·m^{-1}, showed the greatest efficacy as inhibitors of microbial adhesion (Whitekettle, 1991).

Removal of the mature biofilm proved to be a more difficult task. Both the surfactants removed pre-attached bacteria, but total removal of the biofilm did not occur. A significant decrease in the number of adhered bacteria was observed within the first 6 h of treatment. After this initial decrease, the number of attached bacteria stabilised and remained constant. This decrease in attachment could be the result of the removal of the more loosely attached bacteria. The remaining bacteria were irreversibly attached and could not be removed by these compounds at the given concentration. According to Marshall et al. (1971) bacterial adhesion occurs in two phases: reversible sorption where bacteria are held weakly near the surface and are readily removed by washing and irreversible sorption which involves the firm adhesion of the bacteria to the surface and which cannot be removed by washing. Special cell surface structures (e.g. fibrils or polymers) form strong links between the cell and solid surface (Van Loosdrecht et al., 1990). These polymers are essential for the development of surface films (Geesey, 1982). Bacteria that are allowed to adhere strongly and form adhesive polymers (exopolysaccharides) are more difficult to remove (Marshall et al., 1971). A higher concentration of surfactants might therefore be necessary to remove these bacteria.

It is misleading to consider removal of biofilm in terms of percentage (>90%). In real terms, the surfactants resulted in only a 1 log reduction of the actual microbial numbers. In industrial water systems, one seldom starts with a clean surface. Often, biofilms already visible to the naked eye, have to be removed, to prevent biofouling and biocorrosion. This study indicates that the use of a surfactant by itself is not sufficient for controlling biofouling.

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